Plant Physiol. (1988) 86, 0231–0236
0032-0889/88/86/0231/06/$01.00/0

NaCl Induces a Na^+/H^+ Antiport in Tonoplast Vesicles from Barley Roots[1]

Received for publication August 4, 1987 and in revised form October 7, 1987

JOAN GARBARINO* AND FRANCES M. DUPONT
United States Department of Agriculture, Agricultural Research Service, Western Regional Research Center, Albany, California 94710

ABSTRACT

Evidence was found for a Na^+/H^+ antiport in tonoplast vesicles isolated from barley (*Hordeum vulgare* L. cv California Mariout 72) roots. The activity of the antiport was observed only in membranes from roots that were grown in NaCl. Measurements of acridine orange fluorescence were used to estimate relative proton influx and efflux from the vesicles. Addition of MgATP to vesicles from a tonoplast-enriched fraction caused the formation of a pH gradient, interior acid, across the vesicle membranes. EDTA was added to inhibit the ATPase, by chelating Mg^{2+}, and the pH gradient gradually dissipated. When 50 millimolar K^+ or Na^+ was added along with the EDTA to vesicles from control roots, the salts caused a slight increase in the rate of dissipation of the pH gradient, as did the addition of 50 millimolar K^+ to vesicles from salt-grown roots. However, when 50 millimolar Na^+ was added to vesicles from salt-grown roots it caused a 7-fold increase in the proton efflux. Inclusion of 20 millimolar K^+ and 1 micromolar valinomycin in the assay buffer did not affect this rapid Na^+/H^+ exchange. The Na^+/H^+ exchange rate for vesicles from salt-grown roots showed saturation kinetics with respect to Na^+ concentration, with an apparent K_m for Na^+ of 9 millimolar. The rate of Na^+/H^+ exchange with 10 millimolar Na^+ was inhibited 97% by 0.1 millimolar dodecyltriethylammonium.

Salinization of the soil is a major problem on irrigated farmland, and high concentrations of NaCl are often present. Plants that grow successfully in saline soils must maintain a much higher K^+/Na^+ ratio in their cytoplasm than is present in the surroundings (10, 14, 15, 21, 26). Barley, while not considered to be a halophyte, tolerates higher concentrations of NaCl in the soil than most crop plants (10, 12, 14, 15, 21, 32). Transport of ions at the plasma membrane and tonoplast is thought to play an important role in the process by which barley cells maintain a high ratio of K^+/Na^+ in the cytoplasm (13–15, 26, 27, 32). The detailed mechanisms for transport of K^+ and Na^+ across the plasma membrane and tonoplast of barley roots are not known; however, the net effect of the ion transport processes is to selectively take up K^+ into the cytoplasm and to extrude Na^+, both into the vacuole (23, 26) and into the external medium (10, 14, 15).

H^+-ATPases in both the plasma membrane and the tonoplast create proton gradients (29, and references therein) that may serve as energy sources for Na^+ extrusion from the cytoplasm via Na^+/H^+ antiports. Evidence for Na^+/H^+ exchange across both the tonoplast and plasma membrane of salt-tolerant plants has been reported. Niemitz and Willenbrink (24) found evidence for a Na^+/H^+ exchange across the tonoplast of intact vacuoles from red beet roots. They isolated intact vacuoles from red beet protoplasts and measured ATP-dependent acidification of the vacuoles. Although Na^+ did not inhibit ATP hydrolysis, it inhibited formation of a pH gradient. Blumwald and Poole (3) reported that addition of Na^+ to tonoplast vesicles from red beet roots caused a preformed pH gradient to dissipate. They proposed that there was an electrically neutral exchange of Na^+ for H^+, via a Na^+/H^+ antiport. Watad *et al.* (31) demonstrated that tobacco cells responded to the addition of NaCl by an increased proton efflux, and they suggested that a Na^+/H^+ antiport was active in the plasma membrane. Ratner and Jacoby (27) showed that addition of Na^+ increased the proton efflux across the plasma membrane of tobacco cells and barley roots. Katz *et al.* (16) reported the presence of a Na^+/H^+ antiport in plasma membrane vesicles from *Dunaliella salina*, a highly salt-tolerant alga.

Recently, methods to separate the plasma membrane, tonoplast and ER vesicles from barley roots have been developed, and active transport by the tonoplast H^+-ATPase has been characterized in detail (7, 8). Because of the evidence for a Na^+/H^+ exchange in vacuoles and tonoplast vesicles from red beet storage tissue (3), along with the evidence that Na^+ is sequestered in barley vacuoles (23), it seemed likely that a Na^+/H^+ antiport would also be present in tonoplast vesicles obtained from barley roots. Efforts to detect a Na^+/H^+ antiport in tonoplast vesicles from barley roots are described in this paper.

MATERIALS AND METHODS

Plant Material. A salt-tolerant cultivar of barley, *Hordeum vulgare* L. cv California Mariout 72 (2, 21), was grown as described previously (8) above aerated nutrient solution (9) at 22°C for 7 d in the dark. Plants were grown with or without 100 mM NaCl added to the solution.

Membrane Preparations. A membrane fraction enriched in tonoplast was isolated by a method similar to that described previously (8). Barley roots (60 g) were ground in a buffer (400 ml) containing 250 mM sucrose, 50 mM Tris, 8 mM EDTA, and 4 mM DTT. After a 20 min centrifugation at 10,000*g*, the supernatant was centrifuged at 100,000*g* for 35 min. The resulting pellet was layered on a step gradient made up of 9 ml each of 22, 30, 34, and 40% (w/w) sucrose containing 1 mM DTT, 1 mM EDTA, and 1 mM Tris-HCl (pH 7.2). The tonoplast-enriched fraction was collected from the 22/30% sucrose interface. Membranes were diluted with a KCl solution to a final concentration of 150 mM KCl, 2 mM DTT, and 25 mM Tris-HCl (pH 8.0) or with buffer containing 250 mM sorbitol, 1 mM DTT, and 5 mM Mes adjusted to pH 7 with Tris. The membranes were centrifuged at 100,000*g* for 35 min. The KCl-washed membranes were resuspended in 250 mM sucrose, 2 mM DTT, and 5 mM Pipes adjusted to pH 7.2 with KOH. The sorbitol-washed membranes

[1] Funding by United States Department of Agriculture Competitive Grant No. 85-CRCR-1-1647.

were resuspended in the same sorbitol buffer in which they were washed. Enzyme activities were assayed using vesicles suspended in assay buffer containing sorbitol for sorbitol-washed membranes or sucrose for sucrose-washed membranes. H^+-ATPase and Na^+/H^+ exchange activities were similar for vesicles in sucrose or sorbitol. The membranes were frozen at −80°C in 0.5 ml aliquots at a protein concentration of 0.5 to 1.5 mg/ml. Similar enzyme activities were obtained with frozen or freshly prepared membranes.

Assays. A pH gradient was generated across the membrane vesicles by using the activity of the Cl^--stimulated, NO_3^--inhibited H^+-pumping ATPase that is associated with the tonoplast-enriched fraction from barley roots (8). The formation of an acid-interior pH gradient was followed by measuring the quenching of acridine orange fluorescence as described previously (8).

For comparisons of pH gradient formation by the H^+-ATPase in the presence of different salts, the assay buffer contained 250 mM sucrose, 1 mM $MgCl_2$, 1 mM Tris-ATP, 1 mM EGTA, 5 mM Tris adjusted to pH 7.5 with Mes, and 2 μM acridine orange. Salts as indicated were present at a concentration of 50 mM. The assay temperature was maintained at 30°C.

For assays of the rate of recovery of fluorescence after the ATPase was turned off, the assay buffer contained 250 mM sorbitol or sucrose, 1 mM $MgCl_2$, 50 mM choline-Cl, 1 mM EGTA, 2.5 μM acridine orange, and 5 mM Mes adjusted to pH 7.8 with Tris. The assay temperature was maintained at 21°C. Previously we found that addition of valinomycin to the assay buffer dissipated the pH gradient (8). The explanation of this effect is still not clear, but we discovered that valinomycin could be used if 1 mM EGTA was included in the assay buffer. Even with EGTA present, however, addition of 1 μM valinomycin approximately doubled the background proton efflux from the vesicles.

To measure the rate of fluorescence recovery, membranes (10–20 μg protein) were added to 3 ml of assay buffer in a plastic cuvette and formation of a pH gradient was initiated by the addition of 1 mM Tris-ATP. Similar rates of pH gradient formation were observed for membranes from control and from salt-grown roots. After formation of a pH gradient, 2 mM Tris-EDTA was added to chelate the Mg^{2+} and thus halt the activity of the ATPase. The leak of H^+ from the vesicles could then be observed as an increase in fluorescence intensity. The initial rate of recovery of fluorescence (25) was measured and divided by the quench achieved (Q), to give a value for the initial rate of fluorescence recovery expressed as % Q μg protein^{-1} min^{-1}. Q was measured as the fluorescence after addition of 3 mM NH_4Cl minus the fluorescence at maximum quench (Fig. 1). In early experiments the initial rate was estimated by calculating the slope of a line drawn through approximately the first 10 s of the recovery trace. In later experiments data was acquired using a PC-XT computer with the program Asystant+ from Macmillan Software Co.[2] The derivative was taken of the first 20 s of the recovery and the y-intercept of a line fitted through the derivative was used as the initial rate. The rates obtained with the two methods agreed very well. The rate obtained using 2 mM EDTA was subtracted from rates obtained using EDTA plus salt to give the salt-dependent rate of recovery. EDTA was added 2 min after addition of ATP unless otherwise noted.

ATP hydrolysis was measured using assay buffer containing 250 mM sucrose, 1 mM $MgCl_2$, 1 mM Tris-ATP, 1 mM EGTA, 5 mM Tris-Mes (pH 7.5) and 50 mM salts as indicated. The assay time was 30 min. The assay temperature was maintained at 20°C. Pi was measured by the method of LeBel *et al.* (19).

Proteins were assayed by the method of Lowry *et al.* (20).

Synthesis of DoTEA.[3] DoTEA was synthesized (28) by mixing 0.1 mol of triethylamine with 0.1 mol of 1-bromodecane in 75 ml of acetone. The mixture was refluxed for 2 h, then most of the acetone was distilled off. The remaining mixture was placed on ice for 1 to 2 h. The resulting white precipitate was collected on a Buchner funnel, washed with three portions of cold hexane, and dried. Yield was about 5%. Identity and purity of the product was confirmed by mass spectrometry.

Chemicals. Chemicals, including amiloride, were purchased from Sigma Chemical Co.

RESULTS

Generation of pH Gradients. One method to detect a cation/proton exchange in membrane vesicles is to create a pH gradient across the membranes. To detect an inward movement of cations in exchange for an outward movement of protons, a pH gradient, interior acid, is created. Addition of the cation to the external medium should cause the pH gradient to dissipate (3–5, 16).

Several different techniques have been used to create pH gradients. A pH jump method has been used to demonstrate Na^+/H^+ and Ca^{2+}/H^+ exchange in tonoplast vesicles from red beets (3, 4). The vesicles were incubated in a buffer of low pH, diluted into a buffer of higher pH, and the size of the pH gradient that was formed was estimated by the extent of quenching of acridine orange fluorescence. Subsequent addition of Na^+ or Ca^{2+} caused the pH gradient to dissipate, despite the presence of K^+ plus valinomycin to prevent the development of a membrane potential. This method was tried extensively with the barley membranes, but several difficulties were encountered. Conditions of low ionic strength promoted extensive binding of acridine orange to the vesicles, as previously reported for barley plasma membrane vesicles (17). Even in the absence of a pH gradient, a large decrease in fluorescence occurred when the membranes were added to the dye in a buffered solution. Addition of any cations displaced the dye, causing large increases in fluorescence that tended to obscure a subsequent, slower increase in fluorescence that might be attributed to cation/proton exchange (3, 4). Also, there was little difference between the effects of K^+, Na^+, Ca^{2+}, and Mg^{2+} on the second, relatively slow phase of the increase in fluorescence. It is possible that the slow phase of fluorescence increase was due to a large population of 'leaky' vesicles and that it obscured any specific Na^+/H^+ exchange or Ca^{2+}/H^+ exchange in sealed tonoplast vesicles.

An alternative method was therefore used to search for evidence for Na^+/H^+ exchange. This was to use the tonoplast ATPase to generate a pH gradient (Fig. 1). Addition of 1 mM Tris-ATP, in the presence of 1 mM $MgCl_2$ and 50 mM choline Cl, caused a gradual decrease in the fluorescence of acridine orange, indicating that an acid-interior pH gradient was built up by the tonoplast ATPase. The ATPase was then inhibited, by adding EDTA to chelate the Mg^{2+}, and relative rates of proton efflux from the vesicles were estimated by measuring the initial rate of recovery of the acridine orange fluorescence. A standard procedure was to add ATP, wait 2 min for a sufficient pH gradient to build up, and then add 2 mM Tris-EDTA, alone or with other ions. Addition of 2 mM Tris-EDTA, alone or with other ions. Addition of 2 mM Tris-EDTA alone resulted in a relatively slow recovery of fluorescence with vesicles from both control (Fig. 1, A and B) and salt-grown (Fig. 1, C and D) roots. Subsequent addition of 3 mM NH_4Cl caused fluorescence to recover rapidly to near the starting value.

Effect of Na^+ on the Rate of H^+ Efflux from Control and Salt-grown Vesicles. The effect of Na^+ on the rate of fluorescence recovery was tested. There was a significant difference between

[2] Mention of a specific product name by the United States Department of Agriculture does not constitute an endorsement and does not imply a recommendation over other suitable products.

[3] Abbreviations: DoTEA, dodecyltriethylammonium; TEA, tetraethylammonium

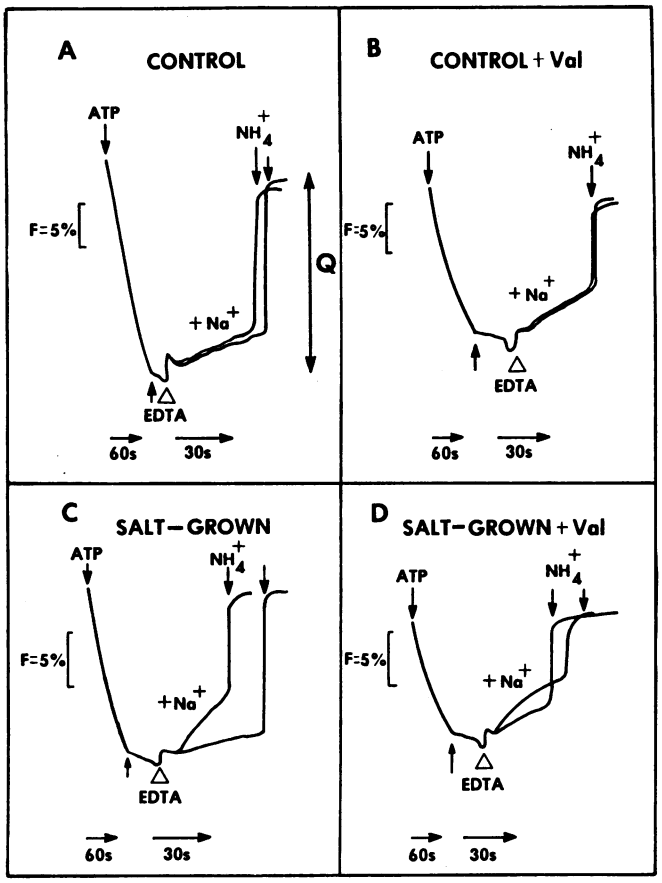

FIG. 1. Effect of Na^+ and EDTA on the rate of dissipation of the ATP-generated pH gradient. Quench and recovery of acridine orange fluorescence was followed as described in "Materials and Methods." Additions of 1 mM ATP and 3 mM NH_4Cl were as indicated. At the time indicated (Δ), 2 mM EDTA or 2 mM EDTA plus 30 mM Na-gluconate were added. Note change in recording speed at small arrow. A, Membranes were from roots grown without NaCl. Protein used per assay was 19 μg. B, Same membrane preparation as in A. Membranes were assayed with 20 mM K-gluconate and 1 μM valinomycin added to the assay buffer. Protein used per assay was 19 μg. C, Membranes were from roots grown in 100 mM NaCl. Protein used per assay was 12 μg. D, Same membrane preparation as in C. Membranes were assayed with 20 mM K-gluconate and 1 μM valinomycin added to the assay buffer. Protein used per assay was 12 μg. The irregularities in the trace at the time of EDTA addition are due to mixing. F is fluorescence, Q is total quench (*i.e.* fluorescence after 3 mM NH_4 minus fluorescence at time of EDTA addition).

the effect of Na^+ on membranes from control roots and the effect of Na^+ on membranes from roots grown in 100 mM NaCl. When the tonoplast vesicles were obtained from control roots, the addition of 30 mM Na-gluconate along with the EDTA gave a small increase over the rate obtained with EDTA alone (Fig. 1A). However, a rapid recovery of fluorescence was observed when Na^+ was added to vesicles from roots that had been grown in 100 mM NaCl (Fig. 1C). The small Na^+-dependent increase with the control vesicles was probably due to Na^+ diffusing into the vesicles down its concentration gradient. This could cause an increase in the membrane potential and thus increase passive H^+ efflux. When 20 mM K-gluconate and 1 μM valinomycin were added to the assay buffer, to clamp the membrane potential at near zero, the rate of recovery with EDTA alone increased (Fig. 1, B and D) but the small amount of Na^+-stimulated recovery with vesicles from the control roots was abolished (Fig. 1B). Addition of K^+ and valinomycin did not eliminate the Na^+-stimulated recovery with vesicles from salt-grown roots (Fig. 1D).

Effect of Na^+ on Generation of the pH Gradient. The effect of Na^+ on generation of a pH gradient by the H^+-ATPase was also tested. Quench was assayed as described in "Materials and Methods" with 50 mM choline-Cl, KCl, or NaCl in the assay buffer. For vesicles from control roots, all three salts gave similar results (Fig. 2A). For vesicles from salt-grown roots, NaCl gave less total quench and a slower rate of quench than did KCl or choline-Cl (Fig. 2B). The effect of K^+ and Na^+ on the rate of ATP hydrolysis was also compared (Table I). Identical rates of total ATPase were obtained whether Na^+ or K^+ was used. Therefore, the differences in Figure 2 were not due to changes in the ATPase rate, but rather to increased dissipation of the H^+ gradient due to Na^+/H^+ exchange.

Effect of Na^+ Concentration on the Fluorescence Recovery. The effect of Na^+ concentration on the Na^+-stimulated fluorescence recovery was tested (Fig. 3). With vesicles from control roots in the absence of K^+ and valinomycin, Na^+ gave a low rate of fluorescence recovery that increased linearly with concentration, as would be expected for a passive, electrochemically coupled exchange. When Na^+ was added to vesicles isolated from salt-grown roots, the initial rate of fluorescence recovery was

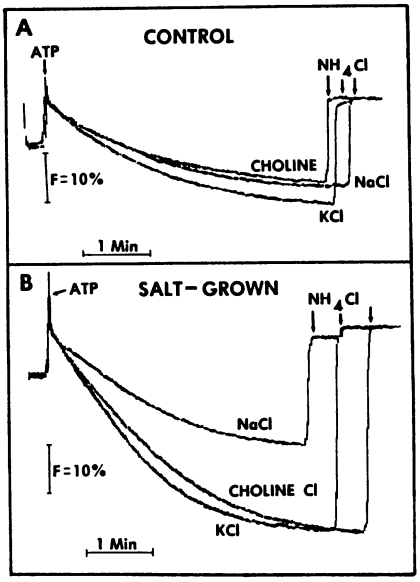

FIG. 2. Fluorescence quench by the tonoplast H^+-ATPase in the presence of various salts. Assays were performed as described in "Materials and Methods," in the presence of 50 mM KCl, NaCl, or choline-Cl as indicated. A, Membranes were from roots grown without NaCl. Protein used per assay was 35 μg. B, Membranes were from roots grown with 100 mM NaCl. Protein used per assay was 23 μg.

Table I. *Effect of Na^+ versus K^+ on Nitrate-Inhibitable ATPase Activity*

Membranes were from roots grown with 100 mM NaCl. Protein used per assay was 18 μg. The assays were performed as described in "Materials and Methods." The salts indicated were added to a final concentration of 50 mM. The results are the average of 3 assays ± SD.

Additions	ATPase
	nmol Pi/mg protein·min
KCl	140 ± 6
KNO_3	66 ± 7
Nitrate inhibition	74 ± 9
NaCl	142 ± 5
$NaNO_3$	69 ± 1
Nitrate inhibition	73 ± 5

approximately 10 times greater than that for the control roots, even when K^+ and valinomycin were included in the assay buffer (Fig. 3). This rapid Na^+-dependent recovery showed Michaelis-Menten kinetics, saturating at about 25 mM, with an apparent K_m for Na^+ of 9 mM. In contrast to Na^+, K^+, in the absence of valinomycin, gave a low rate of fluorescence recovery that was linear with concentration in vesicles from both salt-grown (Fig. 3) and control roots (data not shown). The results for K^+ resembled those obtained when Na^+ was added to vesicles from control roots and may also be due to passive, electrochemically coupled exchange.

The membranes that were used for the experiments in Figures 1 through 3 were from roots that were grown in a full nutrient solution. However, similar effects of sodium were observed when the membranes were obtained from roots grown in 0.5 mM $CaSO_4$ plus or minus 100 mM NaCl. For both full-nutrient and $CaSO_4$-grown roots, no specific Na^+/H^+ exchange was observed unless the roots were grown with NaCl present.

Ion Specificity. The effect of other monovalent cations on the rate of recovery was tested, to determine if the stimulation of the H^+ efflux was specific for Na^+ (Table II). There was no significant stimulation of H^+ efflux by K^+, Li^+, Cs^+, or Rb^+ while NaCl and Na-gluconate both gave large increases.

Effects of Inhibitors. The sensitivity of the Na^+/H^+ exchange to some known inhibitors of K^+ or Na^+ transport was tested (Table III). Generation of a pH gradient was initiated by addition of ATP, as in Figure 1. The inhibitors were added 1.5 min after the ATP, and 10 mM Na^+ plus EDTA was added 0.5 min after the inhibitors. Amiloride is an inhibitor of Na^+/H^+ exchange in many eukaryotes (1, 18) and it has been reported to competitively inhibit the Na^+/H^+ exchange in red beet tonoplast vesicles with a K_i of about 0.1 mM (3). However, addition of 0.5 mM amiloride to barley vesicles gave no significant inhibition of the Na^+/H^+ exchange (Table III).

TEA-Cl has been demonstrated to block Na^+ and K^+ movements in various animal tissues (28 and references therein) and has been reported to block K^+ channels in membranes of *Chara corallina* and *C. australis* (11, 30). At a concentration of 50 mM, TEA-Cl gave 15% inhibition of the Na^+/H^+ exchange (Table III). The inhibition by TEA-Cl was not statistically significant. DoTEA-Br is a derivative of TEA that has been demonstrated to act as a long-lasting anesthetic when injected into the infraorbital nerve of rats (28) or when used to bathe isolated frog nerves (6). It has been presumed to act by blocking cation channels. Addition of 100 μM DoTEA-Br resulted in nearly complete inhibition of the Na^+/H^+ exchange (Table III). The effect of low concentrations of DoTEA on the Na^+/H^+ exchange is shown in Figure 4. Addition of high concentrations (above 0.5 mM) of DoTEA-Br caused the pH gradient to dissipate, probably because the DoTEA-Br acted as a detergent and disrupted the membrane bilayer.

Addition of either amiloride or DoTEA shifted the fluorescence but did not change the value of Q. Both also sometimes

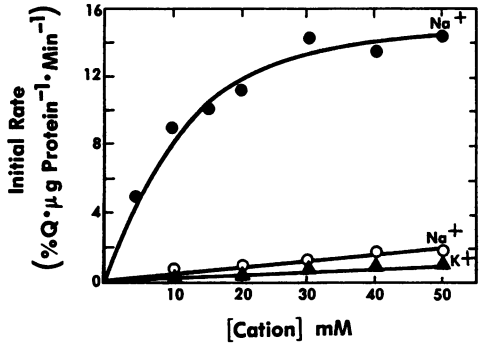

FIG. 3. Initial rate of recovery of fluorescence as a function of cation concentration. The membranes were from roots grown with (●, ▲) or without (○) 100 mM NaCl. Aliquots of Na-gluconate or K-gluconate were added to assay mix containing vesicles in which a pH gradient had been generated as described in "Materials and Methods." The average initial rate of fluorescence recovery observed when EDTA was added without cations was 2.8% Q/μg protein/min (▲), 2.5% Q/μg protein/min (○), or 6.4% Q/μg protein/min (●) and was subtracted from the values in the Figure. Protein used per assay was 12 μg (▲, ●) or 19 μg (○). Assay buffer for (●) contained 20 mM K^+ and 1 μM valinomycin. Each point is the average of two separate experiments.

Table II. *Effect of Various Salts on the Rate of Dissipation of the ATP-Generated pH Gradient*

The initial rate of recovery was measured after addition of the indicated salts to a final concentration of 25 mM. The initial rate of fluorescence recovery with EDTA alone was 4.2 ± 1.3% Q/μg protein/min. Membranes (11 μg protein/assay) were from roots grown with 100 mM NaCl. Results are the average of 3 assays ± SD.

Salts	Fluorescence Recovery
	% Q/μg protein·min
NaCl	9.9 ± 1.9
Na-gluconate	10.7 ± 1.9
KCl	1.3 ± 1.4
K-gluconate	0.3 ± 2.0
LiCl	1.9 ± 2.1
CsCl	1.2 ± 1.9
RbCl	0.8 ± 1.5

Table III. *Effect of Inhibitors on Na^+-Dependent Dissipation of the ATP-Generated pH Gradient*

The indicated compounds were added 1.5 min after ATP and 2 mM EDTA ± 10 mM Na-gluconate was then added at 2 min. The background rate (EDTA without Na^+) has been subtracted. Membranes (7 μg protein/assay) were from roots grown with 100 mM NaCl. The results are the average of three assays ± SD.

Inhibitor	Rate of Recovery	Inhibition
	% Q/μg protein·min	%
No addition	12.5 ± 4.2	
50 mM TEA-Cl	10.6 ± 1.9	15
0.1 mM DoTEA-Br	0.3 ± 2.4	97
0.5 mM amiloride	11.5 ± 2.1	8

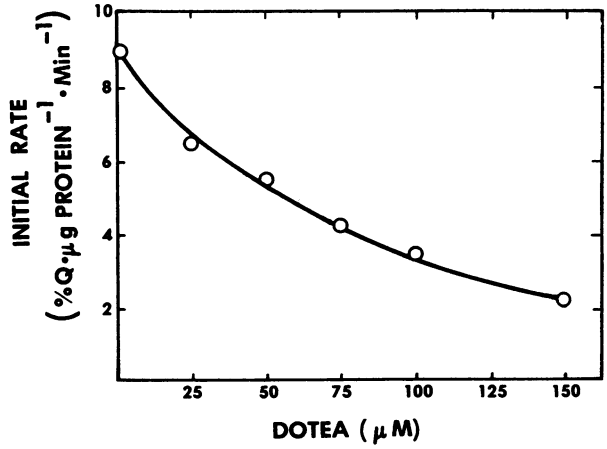

FIG. 4. Inhibition of Na^+/H^+ exchange as a function of DoTEA concentration. DoTEA was added along with 2 mM EDTA and 25 mM Na-gluconate 2 min after ATP addition as described in "Materials and Methods."

increased the rate of recovery seen with EDTA alone, but the increase was not large enough to interfere with measurement of inhibition of the Na^+/H^+ exchange.

DISCUSSION

The barley plant is thought to use multiple mechanisms to cope with the problems of osmotic imbalance, sodium toxicity, and ion imbalance that are brought about when the plant is grown in saline conditions. The end effects of these mechanisms include extrusion of sodium across the plasma membrane of root cortical cells, storage of excess sodium in the vacuoles, partial exclusion of Na^+ from the leaves, redistribution of K^+ from older to younger tissue, and a tendency to concentrate Na^+ in older tissues (10, 14, 15, 21, 26). It is likely that a number of specific molecular mechanisms are responsible for these end results, including several methods of regulating ion transport across the plasma membrane and tonoplast. The results in this paper indicate that one of the mechanisms may be the induction of a Na^+/H^+ antiport in the tonoplast membranes.

The membrane fraction that was used for this study was enriched in tonoplast membranes. When microsomal fractions were centrifuged on sucrose gradients, similar distributions of marker enzymes were observed regardless of whether the membranes were obtained from roots grown in $CaSO_4$ (8), full nutrient solution, or full nutrients plus 100 mM NaCl (WJ Hurkman, CK Tanaka, FM DuPont, manuscript in preparation). The membranes in the tonoplast fraction contained no vanadate-inhibited ATPase, and little NADH Cyt *c* reductase or Cyt *c* oxidase, indicating little contamination by plasma membranes, and only minor contamination by ER and mitochondrial membranes (8). Immunoblots of the tonoplast fraction from barley roots grown in 0.5 mM $CaSO_4$ demonstrated the presence of proteins with cross-reactivity to antibodies to the 72- and 58-kD subunits of the tonoplast ATPase from corn roots (22). We also tested the antigenicity of the tonoplast fraction from roots grown in full nutrient solution plus NaCl, using antibodies to the tonoplast ATPase subunits from red beet storage tissue (gift of A. B. Bennett, UC Davis). The ATPase subunits were greatly enriched in the tonoplast fraction relative to the ER and plasma membrane fractions, whereas antibodies to the plasma membrane ATPase (gift of R. T. Leonard, UC Riverside) reacted principally with the plasma membrane fraction (FM DuPont, WJ Hurkman, CK Tanaka, manuscript in preparation).

The tonoplast fraction did contain a considerable amount of glucan synthase I activity, however, indicating that the tonoplast and Golgi membranes were not well separated. The pH jump method for demonstrating cation/proton exchange probably gave an average of the activities from the tonoplast and Golgi membranes, along with the activity of any other contaminants in the tonoplast fraction, which may explain why that method did not work well for the barley membranes. In contrast, the results from using the ATP-generated pH gradients were obtained only from sealed, right-side out endomembranes containing a H^+-ATPase. The ATP-generated pH gradients were inhibited by KNO_3 and we proposed them to be the activity of a single, tonoplast-type ATPase rather than a mixture of tonoplast and Golgi ATPase (8).

The following evidence supports the contention that a specific Na^+/H^+ antiport was present in tonoplast membranes from roots grown in 100 mM NaCl, and was absent in membranes from control roots. A low rate of K^+- or Na^+-stimulated recovery of fluorescence was observed in vesicles from roots grown without NaCl, but it was not saturable (Fig. 3). It was probably caused by a passive, electrochemically coupled cation/H^+ exchange since addition of K^+ plus valinomycin eliminated the effect (Fig. 1B). The rate of Na^+-stimulated fluorescence recovery in membranes from salt-grown roots was much faster than the rate with membranes from control roots and the Na^+/H^+ exchange was not abolished by the addition of K^+ plus valinomycin. Inclusion of Na^+ in the assay buffer reduced the rate and extent of pH gradient formation by the ATPase (Fig. 2) but had no effect on the rate of ATP hydrolysis (Table I). The Na^+-stimulated H^+ efflux was saturable with respect to Na^+ concentration, with a K_m of approximately 9 mM Na^+, which was similar to that observed for red beet tonoplast (3). Also, the effect was specific to Na^+; no other monovalent cation tested caused significant stimulation of H^+ efflux.

Amiloride inhibited the Na^+/H^+ exchange in tonoplast vesicles from red beet roots with a K_i of 0.1 mM (3) and in *Dunaliela* plasma membrane with a K_i of 0.037 mM (16). However, it had no significant effect on Na^+/H^+ exchange in the vesicles from barley roots at 0.5 mM (Table III). This result is puzzling, because one might assume that the Na^+/H^+ exchange in barley and red beet tonoplast would be very similar.

TEA has been used extensively to block K^+ transport in animal cells (6, 28) and it was reported to block K^+ channels in membranes of *Chara* sp. (11, 30). TEA was also reported to inhibit Na^+ fluxes in nerve cells (28 and references therein). We found no previous references to blocking of Na^+ movements by TEA in plants. The effectiveness of TEA at blocking Na^+ and K^+ channels in animal tissues increased exponentially as one of its side chains was lengthened (6, 28). It was speculated that the mechanism by which the increase in chain length improved the channel blocking was related to the increased hydrophobicity of the inhibitor. A TEA derivative, nonyltriethylammonium, was approximately 10 times more effective than TEA at blocking light-activated K^+ channels in *Chara australis* (30). Similarly, we found that while TEA gave only a small, statistically insignificant inhibition of Na^+/H^+ exchange in the barley vesicles, DoTEA gave nearly complete inhibition at a 500 times lower concentration (Table III).

There is already a considerable body of evidence that the vacuoles of barley roots play an important role in the ability of the plant to tolerate salt better than most glycophytes. Pitman *et al.* (26) reported that the highest ratio of Na^+/K^+ was in the vacuoles of the cortical cells. Jeschke *et al.* (15) found the highest ratios of Na^+/K^+ in the region of cell elongation, which is highly vacuolated, whereas there was a high ratio of K^+/Na^+ in the root meristems, which have only small vacuoles. While barley plants do not fully utilize Na^+ as a vacuolar osmoticum, in the way many halophytes do, barley stores Na^+ in the root vacuoles and thus decreases the flow of Na^+ to the shoot. Thus, salt-tolerant varieties of barley may use a 'partial' halophyte strategy, by sequestering Na^+ in the vacuole of older tissues, especially in the root (14, 26, 32). Leaf vacuoles also accumulate Na^+. Martinoia *et al.* (23) calculated that more than 90% of the Na^+ in protoplasts from barley leaves was found in the vacuoles. California Mariout 72, the cultivar of barley that was used for the studies in this paper, is one of the more salt tolerant cultivars (12, 21, 26). There is evidence that California Mariout has a greater ability to store sodium in the root when compared to more salt-sensitive cultivars. The ratio of Na^+/K^+ was much higher in the roots than in the shoots (21, 32) and more Na^+ was accumulated in root vacuoles of California Mariout than in a more salt-sensitive cultivar of barley (14). California Mariout also was more successful in partitioning Na^+ away from growing tissue than were more salt-sensitive cultivars (21).

In studies using isolated membrane vesicles from plants, Na^+/H^+ antiports have been reported so far only in tonoplast membranes from red beet and sugar beet (3, 5), which are considered to be halophytes, and now from salt-grown roots of a relatively salt-tolerant cultivar of barley. There were several differences between the properties of the Na^+/H^+ antiport in barley and red beet. Compared with red beet, barley was insensitive to amiloride.

Also, the Na^+/H^+ exchange was completely inducible in barley, whereas about half of the activity was constitutive in sugar beet. It has been suggested that the constitutive activity may be a halophyte characteristic (5). It would be interesting to look for the Na^+/H^+ exchange in different varieties of barley and in additional species of plants to determine whether the presence of a Na^+/H^+ antiport is correlated with the degree of salt-tolerance of the plants from which the membranes were obtained.

Na^+/H^+ antiport activity was induced by salt. This may be due to the synthesis of an integral membrane protein or protein complex. The concentration of NaCl used for this study caused specific, quantitative changes in the patterns of proteins synthesized *in vivo* by barley roots (12). Quantitative changes also were observed in the patterns of proteins synthesized *in vitro* from mRNA (W Hurkman, C Fornari, unpublished data). It is possible that one of the proteins whose synthesis is increased by NaCl is the Na^+/H^+ antiporter. However, the data do not rule out other methods of inducing the Na^+/H^+ exchange, such as regulation of an existing membrane protein by phosphorylation or dephosphorylation.

LITERATURE CITED

1. ARONSON PS 1985 Kinetic properties of the plasma membrane Na^+/H^+ exchanger. Annu Rev Physiol 47: 545–560
2. BLOOM A, E EPSTEIN 1984 Varietal differences in salt-induced respiration in barley. Plant Sci Lett 35: 1–3
3. BLUMWALD E, RJ POOLE 1985 Na^+/H^+ antiport in isolated tonoplast vesicles from storage tissue of *Beta vulgaris*. Plant Physiol 78: 163–167
4. BLUMWALD E, RJ POOLE 1986 Kinetics of Ca^{2+}/H^+ antiport in isolated tonoplast vesicles from storage tissue of *Beta vulgaris* L. Plant Physiol 80: 727–731
5. BLUMWALD E, RJ POOLE 1987 Salt tolerance in suspension cultures of sugar beet. Plant Physiol 83: 884–887
6. CURTIS BM, JE SCURLOCK 1981 The mechanism of action of local anesthesia by tetraethylammonium derivatives. Anesthesiology 54: 270–277
7. DUPONT FM, WJ HURKMAN 1985 Separation of the Mg^{2+}-ATPase from the Ca-phosphatase activity of microsomal membranes prepared from barley roots. Plant Physiol 77: 857–862
8. DUPONT FM 1987 Variable effects of nitrate on ATP-dependent proton transport by barley root membranes. Plant Physiol 84: 526–534
9. EPSTEIN E, JD NORLYN 1977 Seawater-based crop production: a feasibility study. Science 197: 249–251
10. GREENWAY H, R MUNNS 1980 Mechanisms of salt tolerance in nonhalophytes. Annu Rev Plant Physiol 31: 149–190
11. HOMBLE F, JM FERRIER, J DAINTY 1987 Voltage dependent K^+-channel in protoplasmic droplets of *Chara corallina*. Plant Physiol 83: 53–57
12. HURKMAN WJ, CK TANAKA 1987 The effects of salt on the pattern of protein synthesis in barley roots. Plant Physiol 83: 517–524
13. JESCHKE WD 1980 Involvement of proton fluxes in the K^+-Na^+ selectivity at the plasmalemma; K^+-dependent net extrusion of sodium in barley roots and the effect of anions and pH on sodium fluxes. Z Pflanzenphysiol 98: 155–175
14. JESCHKE WD 1984 K^+-Na^+ exchange at cellular membranes, intracellular compartmentation of cations, and salt tolerance. *In* RC Staples, ed, Salinity Tolerance in Plants: Strategies for Crop Improvement. John Wiley and Sons, New York, pp 37–65
15. JESCHKE WD, W STELTER, B REISING, R BEHL 1983 Vacuolar Na^+/K^+ exchange, its occurrence in root cells of *Hordeum, Atriplex and Zea* and its significance for K^+/Na^+ discrimination in roots. J Exp Bot 34: 964–979
16. KATZ A, HR KABACK, M AVRON 1986 Na^+/H^+ antiport in isolated plasma membrane vesicles from the halotolerant alga *Dunaliella salina*. FEBS Lett 202: 141–144
17. KÖRNER LE, P KJELLBOM, C LARSSON, IM MOLLER 1985 Surface properties of right side-out plasma membrane vesicles isolated from barley roots and leaves. Plant Physiol 79: 72–79
18. KRULWICH TA 1983 Na^+/H^+ antiporters. Biochem Biophys Acta 726: 245–264
19. LEBEL D, GG POIRIER, AR BEAUDOIN 1978 A convenient method for the ATPase assay. Anal Biochem 85: 86–89
20. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurements with the Folin phenol reagent. J Biol Chem 193: 265–275
21. LYNCH J, E EPSTEIN, A LÄUCHLI 1982 Na^+-K^+ relationships in salt-stressed barley. *In* A Scaife, ed, Proceedings of the IXth International Plant Nutrition colloquim. Commonwealth Agricultural Bureaus, Slough, UK, Vol 2, pp 347–352
22. MANDALA S, L TAIZ 1986 Characterization of the subunit structure of the maize tonoplast ATPase. J Biol Chem 261: 12850–12855
23. MARTINOIA E, MJ SCHRAMM, G KAISER, WM KAISER, U HEBER 1986 Transport of ions in isolated barley vacuoles. Plant Physiol 80: 895–901
24. NIEMIETZ C, J WILLENBRINK 1985 The function of tonoplast ATPase in intact vacuoles of red beet is governed by direct and indirect ion effects. Planta 166: 545–549
25. PERLIN DS, MJD SAN FRANSCISCO, CW SLAYMAN, BP ROSEN 1986 H^+/ATP stoichiometry of proton pumps from *Neurospora crassa* and *Escherichia coli*. Arch Biochem Biophys 248: 53–61
26. PITMAN MG, A LÄUCHLI, R STELZER 1981 Ion distribution in roots of barley seedlings measured by electron probe x-ray microanalysis. Plant Physiol 68: 673–679
27. RATNER A, B JACOBY 1976 Effect of K^+, its counter anion and pH on sodium efflux from barley roots. J Exp Bot 27: 843–852
28. SCURLOCK JE, BM CURTIS 1981 Tetraethylammonium derivatives: ultralong-acting local anesthetics? Anesthesiology 54: 265–269
29. SZE H 1985 H^+-translocating ATPases: advances using membrane vesicles. Annu Rev Plant Physiol 36: 175–208
30. TAZAWA M, T SHIMMEN 1980 Demonstration of the K^+ channel in the plasmalemma of tonoplast-free cells of *Chara australis*. Plant Cell Physiol 21: 1535–1540
31. WATAD AA, P PESCI, L REINHOLD, HR LERNER 1986 Proton fluxes as a response to external salinity in wild type and NaCl-adapted *Nicotiana* cell lines. Plant Physiol 81: 454–459
32. WYN JONES RG, R STOREY 1978 Salt stress and comparative physiology in the Gramineae. IV. Comparison of salt stress in *Spartina X townsendii* and three barley cultivars. Aust J Plant Physiol 5: 839–850